Semaphorin K1

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INTRODUCTION

Field of the Invention

The field of this invention is polypeptides involved in cell guidance.

Background

The semaphorins constitute a large family of evolutionally conserved glycoproteins that are defined by a characteristic semaphorin domain of approximately 500 amino acids (1-3). The first vertebrate semaphorin, collapsin-1 in chick, was identified by its ability to induce growth cone collapse (4). Consistent with this function, its mammalian homologue, sema III, has been shown to repel specific subsets of sensory axons (5). As a result of these and other studies, Coll-1/sema III/D has been implicated in the patterning of sensory axon projections into the ventral spinal cord and cranial nerve projections into the periphery (6-11).

Several other semaphorins have also been implicated as repulsive and/or attractive cues in axon guidance, axon fasciculation, and synapse formation (1, 12-17). In addition, members of semaphorin family have been implicated in functions outside the nervous system, including bone skeleton and heart formation (9), immune function (18, 19), tumor suppression (20-22), and conferring drug resistance to cells (23).

Recent studies have identified the first semaphorin receptor as a member of the neuropilin family. Neuropilin-1 is a high affinity receptor for sema III, E and IV, whereas neuropilin-2 binds differentially to the subfamily of secreted semaphorins (24-27).

The vertebrate semaphorin family can be classified into several phylogenetically distinct subfamilies (15). Each subfamily has a unique structural arrangement of protein domains. The secreted members of the semaphorin family contain a characteristic semaphorin domain at the N-terminus, followed by an immunoglobulin (Ig) domain and a stretch of basic amino acids in the carboxyl-terminal region. Between the N-terminal semaphorin domain and

the transmembrane spanning region, the transmembrane semaphorins contain several alternative structural motifs including either an Ig domain, a stretch of thrombospondin repeats, or a sequence with no obvious domain homology. Interestingly, semaphorin-like sequences have been identified in the genomes of poxviruses (1) and alcelaphine herpesvirus-1 (28), occupying unique branches of the semaphorin phylogenetic tree. Here we report the identification of a GPI-linked human semaphorin -- semaphorin K1 -- which is homologous to the semaphorin encoded by alcelaphine herpesvirus-1 and show that semaphrin K1 polypeptides and nucleic acids are bioactive in modulating nervous and immune system function.

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SUMMARY OF THE INVENTION

The invention provides methods and compositions relating to semaphorin K1 (sema K1) polypeptides, related nucleic acids, polypeptide domains thereof having sema K1-specific structure and activity and modulators of sema K1 function. The polypeptides may be produced recombinantly from transformed host cells from the subject sema K1 polypeptide encoding nucleic acids or purified from mammalian cells. The invention provides isolated sema K1 gene hybridization probes and primers capable of specifically hybridizing with the disclosed sema K1-encoding genes, sema K1-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis (e.g. nucleic acid hybridization screens for sema K1 transcripts), modulating cellular physiology (e.g. by contacting with exogenous sema K1) and in the biopharmaceutical industry (e.g. as immunogens, reagents for isolating other semaphorins, reagents for screening chemical libraries for lead pharmacological agents, etc.).

DETAILED DESCRIPTION OF THE INVENTION

The nucleotide sequence of a natural cDNA encoding a human sema K1 polypeptide is shown as SEQ ID NO:1, and the full conceptual translate is shown as SEQ ID NO:2. The sema K1 polypeptides of the invention include one or more functional domains of SEQ ID NO:2, which domains comprise at least one of (a) SEQ ID NO:2, (b) at least 100 contiguous residues of SEQ ID NO:2, (c) at least 60 contiguous residues of SEQ ID NO:2, residues 340-634, and (d) at least 12 contiguous residues of SEQ ID NO:2, residues 481-634. A cDNA encoding an alcelaphine herpesvirus semaphorin having sequence similarity to the subject sema K1 polypeptides, and its translate are shown as SEQ ID NO:3 and 4, respectively. Sema K1 specific polynucleotides and polypeptides having human sema K1-specific sequences are readily discernable from alignments of the sequences. Preferred sema K1 polypeptides have one or more human sema K1-specific activities, such as cell surface receptor binding and/or binding inhibitory activity and sema K1-specific immunogenicity and/or antigenicity.

Sema K1-specific activity or function may be determined by convenient *in vitro,* cell-based, or *in vivo* assays: e.g. *in vitro* binding assays, cell culture assays, in animals (e.g. gene therapy, transgenics, etc.), etc. Binding assays encompass any assay where the molecular interaction of an sema K1 polypeptide with a binding target is evaluated. The binding target may be a natural extracellular binding target such as a nerve or immune cell surface protein; or non-natural binding target such a specific immune protein such as an antibody, or an sema K1 specific agent such as those identified in screening assays such as described below. Sema K1-binding specificity may be assayed by binding equilibrium constants (usually at least about $10^7 M^{-1}$, preferably at least about $10^8 M^{-1}$, more preferably at least about $10^9 M^{-1}$), by growth cone collapse assays, by the ability to elicit sema K1 specific antibody in a heterologous host (e.g a rodent or rabbit), etc.

For example, deletion mutagenesis is used to define functional sema K1 domains which specifically bind nerve or immune cell surface proteins in cell-based assays described below.

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Table 1. Exemplary sema K1 deletion mutants defining sema K1 functional domains.

Mutant	Sequence	Nerve Cell Binding	Immune Cell Binding
ΔN1	SEQ ID NO:2, residues 8-606	+	+
ΔN2	SEQ ID NO:2, residues 18-606	+	+
ΔN3	SEQ ID NO:2, residues 26-606	+	+
ΔN4	SEQ ID NO:2, residues 39-606	+	+
ΔN5	SEQ ID NO:2, residues 48-606	+	+
ΔC1	SEQ ID NO:2, residues 1-601	+	+
ΔC2	SEQ ID NO:2, residues 1-592	+	+
ΔC3	SEQ ID NO:2, residues 1-584	+	+
ΔC4	SEQ ID NO:2, residues 1-573	+	+
ΔC5	SEQ ID NO:2, residues 1-566	+	+
ΔNC1	SEQ ID NO:2, residues 24-587	+	+
ΔNC2	SEQ ID NO:2, residues 12-568	+	+
ΔNC3	SEQ ID NO:2, residues 41-601	+	+
ΔNC4	SEQ ID NO:2, residues 6-561	+	+
ΔNC5	SEQ ID NO:2, residues 55-605	+	+

In a particular embodiment, the subject domains provide sema K1-specific antigens and/or immunogens, especially when coupled to carrier proteins. For example, peptides corresponding to sema K1- and human sema K1-specific domains are covalently coupled to keyhole limpet antigen (KLH) and the conjugate is emulsified in Freunds complete adjuvant. Laboratory rabbits are immunized according to conventional protocol and bled. The presence of sema K1-specific antibodies is assayed by solid phase immunosorbant assays using immobilized sema K1 polypeptides of SEQ ID NO:2, see, e.g. Table 2.

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Table 2. Immunogenic sema K1 polypeptides eliciting sema K1-specific rabbit polyclonal antibody: sema K1 polypeptide-KLH conjugates immunized per protocol described above.

Sema K1 Polypeptide Sequence	Immunogenicity
SEQ ID NO:2, residues 1-10	+++

SEQ ID NO:2, residues 12-21	+++
SEQ ID NO:2, residues 25-37	+++
SEQ ID NO:2, residues 42-59	+++
SEQ ID NO:2, residues 62-71	+++
SEQ ID NO:2, residues 72-85	+++
SEQ ID NO:2, residues 88-89	+++
SEQ ID NO:2, residues 105-112	+++
SEQ ID NO:2, residues 116-122	+++
SEQ ID NO:2, residues 120-128	+++
SEQ ID NO:2, residues 175-182	+++
SEQ ID NO:2, residues 180-195	+++
SEQ ID NO:2, residues 201-208	+++
SEQ ID NO:2, residues 213-222	+++
SEQ ID NO:2, residues 222-230	+++
SEQ ID NO:2, residues 228-237	+++
SEQ ID NO:2, residues 230-338	+++
SEQ ID NO:2, residues 237-245	+++
SEQ ID NO:2, residues 247-256	+++
SEQ ID NO:2, residues 282-291	+++
SEQ ID NO:2, residues 335-353	+++
SEQ ID NO:2, residues 335-353	+++
SEQ ID NO:2, residues 355-364	+++
SEQ ID NO:2, residues 365-374	+++
SEQ ID NO:2, residues 412-420	+++
SEQ ID NO:2, residues 440-447	+++
SEQ ID NO:2, residues 475-482	+++
SEQ ID NO:2, residues 480-495	+++
SEQ ID NO:2, residues 531-538	+++
SEQ ID NO:2, residues 554-562	+++
SEQ ID NO:2, residues 572-583	+++
SEQ ID NO:2, residues 598-606	+++

The claimed sema K1 polypeptides are isolated or pure: an "isolated" polypeptide is unaccompanied by at least some of the material with which it is associated in its natural state, preferably constituting at least about 0.5%, and more preferably at least about 5% by weight of the total polypeptide in a given sample and a pure polypeptide constitutes at least about 90%, and preferably at least about 99% by weight of the total polypeptide in a given sample. The sema K1 polypeptides and polypeptide domains may be synthesized, produced by recombinant technology, or purified from mammalian, preferably human cells. A wide variety of molecular and biochemical methods are available for biochemical synthesis, molecular expression and purification of the subject compositions, see e.g. Molecular Cloning, A Laboratory Manual (Sambrook, *et al.* Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, *et al.*, Greene Publ. Assoc., Wiley-Interscience, NY) or that are otherwise known in the art.

The invention provides binding agents specific to sema K1 polypeptides, preferably the claimed sema K1 polypeptides, including agonists, antagonists, natural cell surface receptor binding targets, etc., methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. For example, specific binding agents are useful in a variety of diagnostic and therapeutic applications, especially where disease or disease prognosis is associated with improper utilization of a pathway involving the subject proteins. Novel sema K1-specific binding agents include sema K1-specific receptors, such as somatically recombined polypeptide receptors like specific antibodies or T-cell antigen receptors (see, e.g Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory) and other natural binding agents such as Sema K1 cell surface receptors, non-natural intracellular binding agents identified in screens of chemical libraries such as described below, etc. Agents of particular interest modulate sema K1 function, e.g. sema K1-modulatable cellular physiology, e.g. guidance.

Accordingly, the invention provides methods for modulating cell function comprising the step of modulating sema K1 activity, e.g. by contacting the cell with a sema K1 polypeptide, a sema K1 inhibitor, e.g. inhibitory sema K1 deletion mutants, sema K1-specific antibodies, etc. (supra). The target cell may reside in culture or in situ, i.e. within the natural host. The modulator may be provided in any convenient way, including by (i) intracellular expression from a recombinant nucleic acid or (ii) exogenous contacting of the cell. For many in situ applications, the compositions are added to a retained physiological fluid such as

blood or synovial fluid. For CNS administration, a variety of techniques are available for promoting transfer of the therapeutic across the blood brain barrier including disruption by surgery or injection, drugs which transiently open adhesion contact between CNS vasculature endothelial cells, and compounds which facilitate translocation through such cells. Sema K1 polypeptides or polypeptide modulators may also be amenable to direct injection or infusion, topical, intratracheal/nasal administration e.g. through aerosol, intraocularly, or within/on implants e.g. fibers e.g. collagen, osmotic pumps, grafts comprising appropriately transformed cells, etc. A particular method of administration involves coating, embedding or derivatizing fibers, such as collagen fibers, protein polymers, etc. with therapeutic proteins. Other useful approaches are described in Otto et al. (1989) J Neuroscience Research 22, 83-91 and Otto and Unsicker (1990) J Neuroscience 10, 1912-1921. Generally, the amount administered will be empirically determined, typically in the range of about 10 to 1000 μg/kg of the recipient and the concentration will generally be in the range of about 50 to 500 μg/ml in the dose administered. Other additives may be included, such as stabilizers, bactericides, etc. will be present in conventional amounts. For diagnostic uses, the modulators or other sema K1 binding agents are frequently labeled, such as with fluorescent, radioactive, chemiluminescent, or other easily detectable molecules, either conjugated directly to the binding agent or conjugated to a probe specific for the binding agent.

The amino acid sequences of the disclosed sema K1 polypeptides are used to back-translate sema K1 polypeptide-encoding nucleic acids optimized for selected expression systems (Holler et al. (1993) Gene 136, 323-328; Martin et al. (1995) Gene 154, 150-166) or used to generate degenerate oligonucleotide primers and probes for use in the isolation of natural sema K1-encoding nucleic acid sequences ("GCG" software, Genetics Computer Group, Inc, Madison WI). Sema K1-encoding nucleic acids used in sema K1-expression vectors and incorporated into recombinant host cells, e.g. for expression and screening, transgenic animals, e.g. for functional studies such as the efficacy of candidate drugs for disease associated with sema K1-modulated cell function, etc.

The invention also provides nucleic acid hybridization probes and replication / amplification primers having a sema K1 cDNA specific sequence comprising a strand of least one of: (a) SEQ ID NO:1, (b) at least 300 contiguous nucleotides of SEQ ID NO:1, (c) at least 102 contiguous nucleotides of SEQ ID NO:1, nucleotides 1017-2498, and (d) at least 36 contiguous nucleotides of SEQ ID NO:1, nucleotides 1441-2498, and sufficient to

specifically hybridize with a second nucleic acid comprising the complementary strand of SEQ ID NO:1. Demonstrating specific hybridization generally requires stringent conditions, for example, hybridizing in a buffer comprising 30% formamide in 5 x SSPE (0.18 M NaCl, 0.01 M $NaPO_4$, pH7.7, 0.001 M EDTA) buffer at a temperature of 42°C and remaining bound when subject to washing at 42°C with 0.2 x SSPE; preferably hybridizing in a buffer comprising 50% formamide in 5 x SSPE buffer at a temperature of 42°C and remaining bound when subject to washing at 42°C with 0.2 x SSPE buffer at 42°C.

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Table 3. Exemplary sema K1 nucleic acids which hybridize with a strand of SEQ ID NO:1 under Conditions I and/or II.

sema K1 Nucleic Acids	Hybridization
SEQ ID NO:1, nucleotides 1-36	+
SEQ ID NO:1, nucleotides 68-98	+
SEQ ID NO:1, nucleotides 95-130	+
SEQ ID NO:1, nucleotides 175-220	+
SEQ ID NO:1, nucleotides 261-299	+
SEQ ID NO:1, nucleotides 274-310	+
SEQ ID NO:1, nucleotides 331-369	+
SEQ ID NO:1, nucleotides 430-470	+
SEQ ID NO:1, nucleotides 584-616	+
SEQ ID NO:1, nucleotides 661-708	+
SEQ ID NO:1, nucleotides 789-825	+
SEQ ID NO:1, nucleotides 928-965	+
SEQ ID NO:1, nucleotides 1017-1043	+
SEQ ID NO:1, nucleotides 1053-1072	+
SEQ ID NO:1, nucleotides 1073-1095	+
SEQ ID NO:1, nucleotides 1096-1113	+
SEQ ID NO:1, nucleotides 1132-1152	+
SEQ ID NO:1, nucleotides 1238-1255	+
SEQ ID NO:1, nucleotides 1275-1295	+
SEQ ID NO:1, nucleotides 1380-1400	+
SEQ ID NO:1, nucleotides 1430-1450	+

SEQ ID NO:1, nucleotides 1476-1498	+
SEQ ID NO:1, nucleotides 1545-1577	+
SEQ ID NO:1, nucleotides 1631-1654	+
SEQ ID NO:1, nucleotides 1765-1790	+
SEQ ID NO:1, nucleotides 1812-1833	+
SEQ ID NO:1, nucleotides 1944-1959	+
SEQ ID NO:1, nucleotides 2003-2021	+
SEQ ID NO:1, nucleotides 2121-2143	+
SEQ ID NO:1, nucleotides 2232-2250	+
SEQ ID NO:1, nucleotides 2378-2397	+
SEQ ID NO:1, nucleotides 2480-2498	+

The subject nucleic acids are of synthetic/non-natural sequences and/or are isolated, i.e. unaccompanied by at least some of the material with which it is associated in its natural state, preferably constituting at least about 0.5%, preferably at least about 5% by weight of total nucleic acid present in a given fraction, and usually recombinant, meaning they comprise a non-natural sequence or a natural sequence joined to nucleotide(s) other than that which it is joined to on a natural chromosome. Recombinant nucleic acids comprising the nucleotide sequence of SEQ ID NO:1, or requisite fragments thereof, contain such sequence or fragment at a terminus, immediately flanked by (i.e. contiguous with) a sequence other than that which it is joined to on a natural chromosome, or flanked by a native flanking region fewer than 10 kb, preferably fewer than 2 kb, which is at a terminus or is immediately flanked by a sequence other than that which it is joined to on a natural chromosome. While the nucleic acids are usually RNA or DNA, it is often advantageous to use nucleic acids comprising other bases or nucleotide analogs to provide modified stability, etc.

The subject nucleic acids find a wide variety of applications including use as translatable transcripts, hybridization probes, PCR primers, diagnostic nucleic acids, etc.; use in detecting the presence of sema K1 genes and gene transcripts and in detecting or amplifying nucleic acids encoding additional sema K1 homologs and structural analogs. In diagnosis, sema K1 hybridization probes find use in identifying wild-type and mutant sema K1 alleles in clinical and laboratory samples. Mutant alleles are used to generate allele-specific oligonucleotide (ASO) probes for high-throughput clinical diagnoses. In therapy, therapeutic

sema K1 nucleic acids are used to modulate cellular expression, concentration or availability of active sema K1.

The invention provides efficient methods of identifying agents, compounds or lead compounds for agents active at the level of a sema K1 modulatable cellular function. Generally, these screening methods involve assaying for compounds which modulate sema K1 interaction with a natural sema K1 binding target. A wide variety of assays for binding agents are provided including labeled *in vitro* protein-protein binding assays, immunoassays, cell based assays, etc. The methods are amenable to automated, cost-effective high throughput screening of chemical libraries for lead compounds. Identified reagents find use in the pharmaceutical industries for animal and human trials; for example, the reagents may be derivatized and rescreened in *in vitro* and *in vivo* assays to optimize activity and minimize toxicity for pharmaceutical development.

The following experimental sections / examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Cloning of Sema K1. Four human ESTs, R33537, W47265, R33439, H03806, and one mouse EST, AA260340, were identified that show highest homology with the semaphorin gene in alcelaphine herpesvirus-1 (AHV sema). Oligos corresponding to the sequences of human ESTs were used to amplify by PCR a cDNA fragment from a human testis cDNA library (GIBCO BRL). This PCR fragment corresponds to the central portion of sema K1. The 3' end was cloned by rapid amplification of cDNA ends (RACE) using human placenta Marathon-Ready cDNA from Clontech (29). The remaining 5' end was cloned by PCR amplification from a Clontech human brain λgt11 cDNA library using an internal primer from sema K1 and an anchor primer corresponding to the λgt11 vector sequence. A specific PCR product corresponding to the 5' end was identified by Southern Blot using sema K1 oligos as probes. The full length cDNA of human sema K1 except the region corresponding to the signal peptide sequence was independently cloned from Clontech human placenta λgt10 library by high fidelity PCR amplification and its DNA sequence reconfirmed.

Expression Constructs. Three expression constructs were made that allow the expression of recombinant proteins tagged with either a myc-his tag at the carboxyl terminus (pEX-mh), an alkaline phosphatase tag at the amino terminus and a myc-his tag at the

carboxyl terminus (pEX-AP), or an Fc domain of human immunoglobulin at the carboxyl terminus (pEX-Fc). Similar expression constructs have been made for collapsins and semaphorins and the resulting fusion proteins were shown to be fully functional (7, 10, 23, 24, 30, 31). The multiple cloning site of pSecTagA (Invitrogen) was excised with Pme I and Nhe I and cloned into pcDNA3.1 (Invitrogen) to make myc-his tagged vector pEX-mh. This expression vector contains a signal peptide sequence from immunoglobulin kappa chain for protein secretion. The cDNA for human placental alkaline phosphatase was PCR amplified from pSEAP (Clontech) and cloned into the SfiI site of pEX-mh maintaining the original reading frame to make the AP-tagged vector pEX-AP. The Fc domain of human IgG1 and an enterokinase cleavage site were PCR amplified from Signal-pIgplus (Novagen) and cloned into the Apa I to Pme I sites of pEX-mh maintaining the original reading frame to make the Fc-tagged vector pEX-Fc. Various cDNAs for full length sema K1, extracellular domain of sema K1 (residues starting from Gly-612 to the carboxyl terminal end were deleted), sema III, and neuropilin-1 were PCR amplified from cDNA libraries and subcloned into these expression vectors. The neuropilin-2 expression construct was as previously described (25).

Cell Surface Staining. COS-7 cells were transiently transfected with the full length sema K1 in pEX-AP vector using lipofectamine (GIBCO-BRL). Two days after transfection, cells were washed and treated with or without PI-PLC (Boehringer Mannheim) at 250 mU/ml for 1 hour at 37 °C. Cells were then fixed in 4% paraformaldehyde for 10 min at room temperature. After PBS wash, cells were incubated with a rabbit anti-AP antibody (Accurate Antibodies) at a dilution of 1:500 for one hour followed by a Cy3-anti-rabbit antibody at a dilution of 1:200. The fluorescent images of the transfected cells were photographed in a Zeiss microscope using a 40x lens.

Western Blotting. COS-7 cells were transiently transfected with the full length sema K1 in pEX-AP vector with Lipofectamine (GIBCO-BRL). Cells transfected with the full length CD100 in pEX-AP served as a control. Two days after transfection, cells were incubated with or without 250 mU/ml of PI-PLC (Boehringer Mannheim) for 1 hour at 37 °C. Supernatants and cell lysates were collected and run on a 4-20% SDS-PAGE gel and the AP-tagged sema K1 protein was detected with a HRP-conjugated anti-alkaline phosphatase antibody.

Protein Expression. Stable 293 cell lines secreting myc-his tagged, AP-tagged, or Fc-tagged sema K1 and sema III were derived from transfection of various expression

plasmids followed by G418 selection. Conditioned media from stably transfected cell lines were collected and were confirmed for the expression and integrity of recombinant proteins by Western Blot using anti-AP, anti-Fc, or anti-myc antibodies. SDS-PAGE gel demonstrated that sema K1-Fc fusion protein migrates as a dimer linked by the disulfide bonds in the Fc region, while the sema K1-mh and AP-sema K1 are monomeric. Approximately equal amount of AP- or Fc- tagged sema III and sema K1 fusion proteins as judged by Western Blot were used in the ligand binding experiments. The amount of active sema III used for the ligand binding experiment was further quantified by a growth cone collapse assay and estimated to be over 80 collapsing units/ml (4, 7).

Ligand Binding Experiments. COS-7 cells were transiently transfected with full length neuropilin-1 or neuropilin-2 expression constructs with FuGENETM 6 (Boehringer Mannheim). The expression of neuropilin-1 or -2 was confirmed using a monoclonal antibody 9E10 against the myc tag at the carboxyl terminal ends of both receptors. After two days of transfection, the cells were then incubated with supernatants containing approximately equal amount of sema III-Fc or sema K1-Fc for 1 hour. After post-fixing in 1% paraformaldehyde for 10 min, the cells were heat-inactivated at 65 °C for 1 hour to destroy the endogenous alkaline phosphatase activity. Cells were then incubated with alkaline phosphatase-conjugated anti-Fc antibody at 1:500 dilution for 1 hour and processed for chromogenic AP enzymatic reaction.

For the immune cell staining experiment, P388D1 or RBL-2H3 cells were fixed in 1% paraformaldehyde for 10 min. The suspension cells (A20 and Jurkat) were washed in PBS once and fixed in 1% paraformaldehyde for 10 min and then cytospun onto glass slides. After blocking for 30 min, AP-sema K1 or AP-sema III containing supernatants were added to each well and incubated for 1 hour. The cells were then post-fixed in 100% methanol for 10 min, and the endogenous AP activity was heat-inactivated at 65 °C for 1 hour. Cells were then processed for chromogenic AP enzymatic reactions. AP alone was used as a negative control. For experiments in which sema K1-mh or sema III-mh were used to compete with AP-sema K1 or AP-sema III binding, respectively, sema K1-mh or sema III-mh was incubated with different cell lines for 30 minutes at room temperature prior to AP-sema K1 or AP-sema III incubation.

In Situ Hybridization. A 298 bp DNA fragment corresponding to the sequence of mouse EST AA260340 was PCR amplified from a mouse cDNA library. This DNA fragment

is predicted to encode a mouse homologue of human sema K1 based on the fact that it shares over 95% amino acid identity with the corresponding region of human sema K1. It was used as a probe in the in situ hybridization experiments. In situ hybridization procedure was performed on cryostat sections of E11, E15 mouse embryos and on brain and spinal cord sections of P3 and 5 week old mice as described (32). Tissues were fixed in 4% paraformaldehyde for four hours at 4 °C and embedded in OTC embedding compound. 20 ?m sections were cut and were treated with 1.0 μg/ml proteinase K for 15 min at 37 °C, 0.2 M HCl for 20 min, and then acetylated for 10 min with 0.1M triethanolamine and 0.25% acetic anhydride. Sections were prehybridized for one hour at 65 °C, then hybridized with digoxigenin-labeled probes (2 μg/ml) overnight at 55 °C. The hybridization buffer consists of 50% formamide, 5X SSC, 10% dextran sulfate, 1X Denhardt's, 0.25 mg/ml tRNA, 0.1 mg/ml ssDNA. After hybridization, slides were washed with 0.2xSSC for 60 min at 65 °C and detected with an AP-conjugated anti-digoxigenin antibody at a dilution of 1:2000.

Semaphorin K1 is highly homologous to a viral semaphorin. In an effort to identify veterbrate homologues of viral semaphorins, we have searched existing EST databases against semaphorin-like sequences found in vaccinia virus and in alcelaphine herpesvirus-1 using the BLAST algorithm (33). Four human and one mouse ESTs were identified, which encode novel sequences that were most homologous to the semaphorin gene in alcelaphine herpesvirus-1 (AHV sema, 28). PCR primers were designed based on the EST sequences and were used to obtain a 2.5 kb cDNA that encodes a candidate semaphorin gene. The cDNA contains all the human EST sequences and encodes a protein of 634 amino acids with a molecular mass of 71.5 kDa. This protein is named semaphorin K1 (sema K1). Hydropathy analysis of the sema K1 sequence (34) indicates that the sema K1 sequence lacks approximately half of the signal peptide sequence required for protein secretion (35). Consistently, the alignment between AHV sema and sema K1 also showed an eight amino acid difference at the amino terminal end of sema K1. The hydropathy analysis also identified a long stretch of hydrophobic residues at the carboxyl-terminal end, a signal peptide sequence required for GPI-anchorage (36). This sema K1 protein represents a paradignmatic GPI-linked membrane protein in the semaphorin family.

The sequence of sema K1 is closely related to that of AHV sema. While 50% of amino acid identities are shared between the sema domains of sema K1 and AHV sema, less than 30% of amino acid identities are shared between the sema domains of sema K1 and

other known semaphorins. In addition, 17 out of 18 cysteine residues and 4 out of 5 potential N-linked glycosylation sites are conserved. The homology extends throughout the entire amino acid sequences of sema K1 and AHV sema except at the carboxyl-terminal end, where only sema K1 contains the signal peptide sequence for GPI-anchorage. Thus, sema K1 appear to be a GPI-anchored membrane protein while AHV sema is a secreted protein. The unique structural arrangement of sema K1 defines a new subfamily of vertebrate semaphorins. Consistently, protein sequence homology analysis showed that sema K1 and AHV sema belong to the same branch of the dendrogram tree and this branch is distinct from that of other semaphorins. Sequence alignment with other semaphorins also revealed that members of the viral-related semaphorin subfamily lack three tryptophan residues conserved in other semaphorins, indicating a structurally distinct viral sema domain.

Semaphorin K1 is a GPI-anchored membrane protein. To confirm that sema K1 is a GPI-anchored membrane protein, we have transfected COS-7 cells with a sema K1 expression construct and determined the localization of the expressed sema K1 protein. In order to track sema K1 protein expression, an AP-tagged version of sema K1 was engineered in which the human placenta alkaline phosphatase was fused to the full length sema K1 at the N-terminus. This fusion protein can be detected with an anti-AP antibody. Upon transfection of the expression construct into COS-7 cells, the sema K1 fusion protein was detected on the surface of those transfected cells. Treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) resulted in a complete removal of the fusion protein from cell surfaces. To examine whether the release of sema K1 fusion protein from cell surfaces is a specific action of PI-PLC rather than the result of random proteolysis, we compared the presence of this fusion protein in the supernatant and lysate of transfected COS-7 cells with or without PI-PLC treatment. Supernatants and lysates from PI-PLC treated or untreated cells were subjected to Western Blot analysis. A 150 kDa protein corresponding to the predicted size of the fusion protein was detected with the anti-AP antibody. When the transfected COS-7 cells were not treated with PI-PLC, most, if not all, of the fusion protein was found to be associated with the cell lysate. Treatment of these cells with PI-PLC resulted in significant release of the fusion protein from the cell lysate into the supernatant, without apparent proteolysis. In a control experiment, PI-PLC treatment did not release the transmembrane semaphorin CD100 into the cell supernatant. Furthermore, when a stop codon was introduced immediately N-terminal to the predicted signal peptide sequence for GPI-linkage,

the resultant sema K1 protein was released to the cell supernatant (see below). Thus, we conclude that sema K1 is attached to the cell membrane via a GPI linkage.

Semaphorin K1 binds to specific immune cell lines. Neuropilin-1 and neuropilin-2 have recently been identified as receptors or components of a receptor complexes for sema III and other secreted semaphorins (24-26). To determine whether sema K1 could use neuropilin-1 or -2 as its receptor, we tested the ability of sema K1 to bind COS-7 cells transfected with neuropilin expression constructs. Soluble sema K1 fusion proteins containing either an AP tag at the N-terminus (AP-sema K1), an Fc domain of human IgG1 at the C-terminus (sema K1-Fc), or a myc-his tag at the C-terminus (sema K1-mh) were produced and were used in the ligand binding assay. Similarly arranged AP-sema III, sema III-Fc, and sema III-mh fusion proteins were prepared as controls. To test for interactions with neuropilin-1 or -2, sema K1-Fc or AP-sema K1 were incubated with neuropilin-expressing COS-7 cells, and ligand binding was detected using an anti-Fc antibody or a chromogenic AP enzymatic reaction. Under conditions where sema III-Fc binds to COS-7 cells expressing neuropilin-1 or -2, the dimerized sema K1-Fc does not bind to either (note that sema III binds to neuropilin-2 with lower affinity than to neuropilin-1). Similarly, under conditions when AP-sema III can bind to COS-7 cells expressing neuropilin-1 or -2, the monomeric AP-sema K1 does not bind to these cells. Thus, sema K1 does not bind neuropilin-1 or -2 with high affinity, and may not act through these receptors.

To determine whether or not the soluble sema K1 fusion proteins are competent to bind a cognate receptor and to provide an entry point for investigating the role of sema K1 in modulating immune function, we analyzed several immune cell lines for the presence of sema K1 binding sites. AP-sema K1 or AP-sema III were incubated with Jurkat T cells, A20 B cells, P388D1 macrophages, and RBL-2H3 mast cell lines and the bound ligands were detected with chromogenic AP enzymatic reaction. AP-sema K1 bounds only to the cell surfaces of P388D1 macrophage and RBL-2H3 mast cell lines. This binding is specific, since AP alone does not bind to any of the cell lines and the binding could be competed by preincubation with sema K1-mh. In comparison, AP-sema III binding was detected on cell surfaces of all four immune cell lines tested. This binding is also specific, since preincubation of these cells with sema III-mh blocks the binding. The ability of sema III-Fc or sema K1-Fc to bind these four cell lines was also tested and similar results obtained. We conclude that sema III can bind the four immune cell lines tested, which contrasts with the more selective

binding of sema K1 to macrophage and mast cell lines, suggesting the existence of a specific receptor for sema K1 in these cell lines.

Semaphorin K1 is preferentially expressed in postnatal and adult brain and spinal cord. In order to help define the biological role of sema K1, we examined the expression of sema K1 by Northern blot analysis and in situ hybridization. A 298 bp cDNA corresponding to the mouse homologue of human sema K1 was used as a probe in these studies. This probe does not cross-hybridize with the mRNA of other semaphorins. Northern blot analysis of mRNA isolated from adult mouse tissues revealed a single sema K1 transcript at 4.4 kb. The sema K1 transcript is highly expressed in brain, spinal cord, lung, and testis; moderately expressed in heart, muscle, adrenal gland, lymph nodes, thymus, and intestine; weakly expressed in spleen and kidney; and not detectable in liver, bone marrow, and stomach.

To examine the distribution of sema K1 mRNA in detail, in situ hybridization analysis was performed on tissue sections of embryonic day 11 and day 15 embryos, and on the brain and spinal cord sections of postnatal day 3 and 5 week old mice. A digoxigenin-labeled antisense RNA probe for sema K1 was used in this study. The sema K1 sense probe served as a control, which gave no significant hybridization signal on tissue sections of P3 and adult mice, but gave weak and uniform background signals in E11 and E15 tissue sections. Sema K1 mRNA does not appear to express significantly in the developing mouse embryo since no strong hybridization signals were detected in tissue sections generated from entire E11 and E15 embryos. Above background hybridization signal was detected in the ventral and lateral regions of the spinal cord at E11 and E15. At P3, the signal became more intense and expanded both dorsally and medially. By 5 weeks, strong hybridization signals were present in cells scattered throughout the gray matter except in the dorsal region where Rexed lamina layer I and II reside.

No significant expression of sema K1 mRNA is detected at E11 and E15 in the primodial cerebral cortex and cerebellum. At P3, intense expression of sema K1 mRNA become evident in the marginal zone of the cerebral neocortex. Moderate levels of expression were detected in the cortical plate and subplate. In the brain of 5 week old mice, the expression of sema K1 mRNA becomes widespread throughout the entire cerebral cortex. The level of mRNA expression is moderate among all lamina layers except layer I, where no expression is evident. In the cerebellum at P3, sema K1 message is strongly expressed in the external germinal layer and the primodial Purkinje cell layer. By 5 weeks, intense expression

of sema K1 mRNA is found only in the Purkinje cells. In addition to the dynamic patterns of expression in spinal cord, cerebellum, and cortex, sema K1 mRNA is found to be present in other structures of adult brain, including the cochlear nucleus, inferior colliculus, hippocampus and dentate gyrus, the olfactory glomerular cell layer and mitral cell layer, and thalamic structures.

In vivo activity of sema K1 polypeptides. Rats (12 animals) receive a unilateral lesion of the nucleus basalis by infusion of ibotenic acid. Two weeks after the lesion, osmotic minipumps are implanted, that infuse 1 microgram human recombinant FLAGG-tagged dominant negative sema K1 polypeptide (SEQ ID NO:2, residues 180-634) per day into the lateral ventricle essentially as described in Andrews TJ, et al. (1994) J Neurosci 14(5 Pt 2):3048-3058 . A second group of rats (12 animals) is subjected to fluid-percussion brain injury alone followed by sema K1 infusion, essentially as described in Sinson G, et al. (1997) J Neurosurg 86(3):511-518. After two weeks of treatment, immunohistochemical analysis of cerebral sections reveal that exogenous sema K1 polypeptides enhance organotypic neurite outgrowth from damaged neurons undergoing nerve fiber atrophy.

In vivo activity of antisense sema K1 nucleic acids. Antisense oligonucleotides directed against sema K1 mRNA are administered intracerebroventricularly to twelve rats daily for two weeks substantially as described in Wan HZ, et al. (1998) J Nutr 128(2):287-291. Another twelve rats are administered intracerebroventricularly with missense oligonucleotides as controls. Immunohistochemical analysis of cerebral sections reveal significantly enhance neurite outgrowth and axon formation in the animals treated with the antisense oligonucleotides.

In vivo activity of anti-sema K1 antibodies. Anti-sema K1 antibodies are injected intraventricularly into eight rats and eight guinea pigs essentially as described in Costa M, et al. (1979) Brain Res 173(1):65-78. Immunohistochemical analysis of cerebral sections reveal that injection of anti-sema K1 antibodies inhibits degeneration of and enhances axon outgrowth from cerebral neurons in both rats and guinea-pigs. In rats it is necessary to infuse exogenous complement in the form of guinea-pig serum together with the anti-sema K1, whereas in guinea-pigs the anti-sema K1 is effective on its own.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Although the

foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.